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Chest (1982) 82(2) p239 Saltini et al.

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(54) Detecting or quantifying multiple analytes

(57) A method for the assay, detection etc of each of a plurality of substances of interest in a sample comprises labelling each of the substances with one or more components each capable of taking part in a respective distinguishable chemiluminescent reaction. Luminescent reagents for use in the method are also disclosed.

At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.

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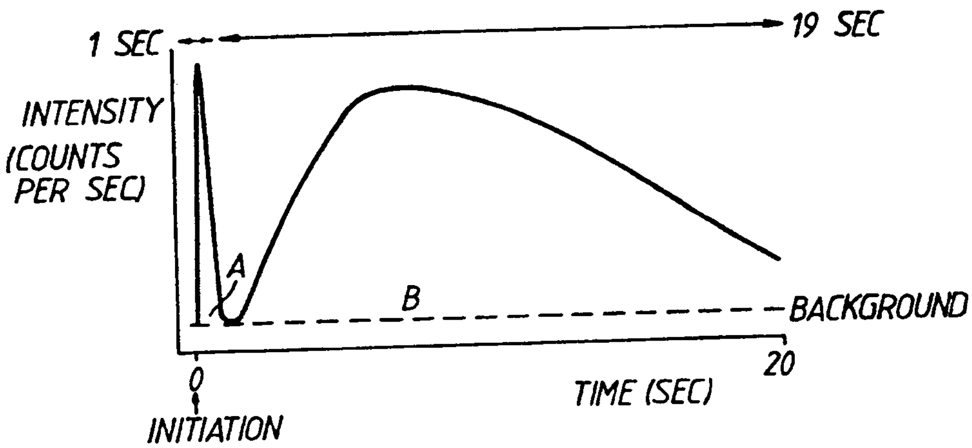
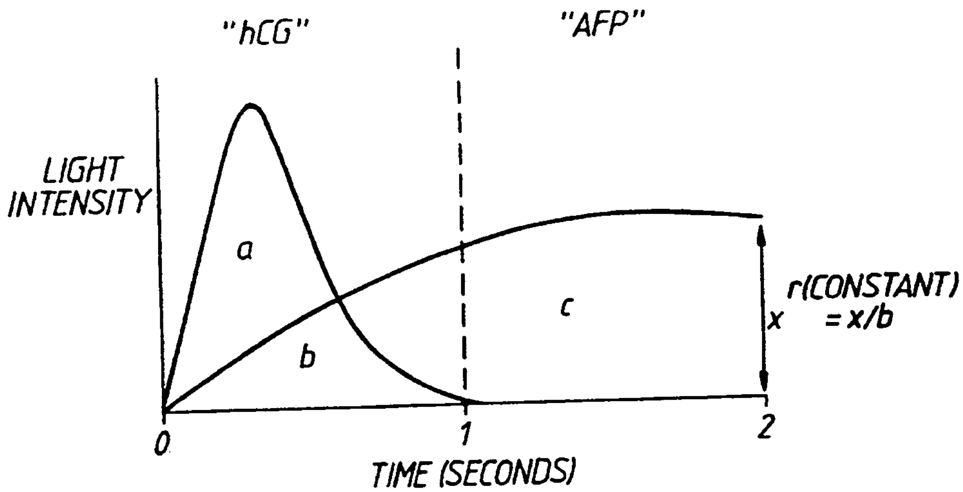


Fig.1.



AFP SIGNAL = c

APPARENT hCG SIGNAL = $(a + b)$

TRUE hCG SIGNAL = $(a + b) - x/r$

Fig.2.

Detecting or Quantifying Multiple Analytes using labelling techniques

This invention relates to methods and reagents for the assay, detection, quantification, location or analysis of each of a plurality of substances of interest ("analytes") in a sample in which each substance is linked ("labelled" with) another molecule or molecules capable of taking part in a chemiluminescent reaction.

For the purposes of this specification, a chemiluminescent reaction is defined as one which involves a chemical reaction that results in the emission of electromagnetic radiation. This luminescence is to be distinguished clearly from fluorescence and phosphorescence.

A luminescent reaction is normally one between at least two molecules (S and L) with or without other reagents, cofactors, or a catalyst (D) or under the influence of a physical trigger. L is the substance which generates light, such as luminol. S is the substance which reacts with L to cause excitation, for example oxygen or hydrogen peroxide. D (if present) is a cofactor, and/or catalyst or trigger such as an enzyme, a luciferase, or potassium ferricyanide. The reaction between L and S results in the conversion of L to an excited molecule L* and the return of this excited molecule to a non-excited state results in the emission of a photon. The reaction between L and S and

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the decay of L* to the non-excited state may take place spontaneously or may require the presence of the cofactor or catalyst D, or a physical trigger such as temperature. An example of such a reaction is the oxidation by H_2O_2 of luminol. The catalyst and cofactors are often inorganic compounds as here, but may also be extracted from biological material such as the enzyme peroxidase which catalyses the luminescent reaction involving luminol.

These methods and reagents discussed above may be used in a wide variety of techniques such as immuno-assays, protein binding assays, nucleic acid hybridisation assays, cellular receptor binding assays and other analogous techniques which involve binding of the substance of interest with a specific binding partner or reagent. These types of linking are referred to herein as "binding or otherwise linking with".

The substances of interest may be peptides, proteins, polypeptides, nucleic acids and other substances of biological interest.

Binding assays have been used for many years in the quantitation of molecules of biological interest. Numerous examples have been described in which the binding step is an immunological reaction, a protein binding reaction, reaction with a cellular receptor or a complementary nucleic acid hybridisation reaction. Sensitive assays based on these reactions require the use

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of a labelwhich can be attached or incorporated into one of the binding partners of such a reaction such that the degree of binding and hence the concentration or mass of another component of the reaction - the substance of interest - can be determined. Many variations of the basic binding reactions have been described and many different labels used, including radioisotopes, enzymes, fluorescent molecules and chemiluminescent molecules.

Various combinations of these have been used in sequence for the detection and quantitation of a wide variety of analytes ranging from small molecules such as hormones and drugs to large molecules such as nucleic acids.

Generally speaking, these techniques have only been applied to the investigation of a single analyte in one test reaction, but there have been a limited number of examples where two analytes have been determined essentially using a single test procedure. The best known of these have been simultaneous immunoassays and/or protein binding assays for vitamin B12 and folic acid and also for thyroxine and thyrotrophin. In these cases the two different reactions are monitored independently using a different radioactive isotope for each. Here use is made of cobalt-57 and iodine-125 whose radioactive emissions are distinguishable using an appropriate gamma counter. Similar strategies have also been used for

the simultaneous determination of lutrophin and follitrophin.

Radioactive reagents have three major disadvantages. Firstly, the method of labelling involves the use of highly radioactive and hence potentially hazardous reagents. Secondly, the shelf life of the radioactively labelled substance is often relatively short not only because by its very nature the radioactive isotope is continuously decaying, but also radioactively labelled proteins are often unstable. Thirdly, it is often difficult to label proteins sufficiently to provide a sensitively and rapidly detectable reagent. The measurement of luminesence is both highly sensitive and very rapid, the time of measurement being of the order of seconds rather than the several minutes normally required for measurement of radioactivity. The attachment either covalently or non-covalently, to substances not normally capable of taking part in a luminescent reaction of a substance which is capable of taking part in a luminescent reaction provides a reagent which can be rapidly measured in very small quantities.

Work has been described relating to the use of different fluorescent molecules in so-called "dual labelling" systems. However, fluorescent labelling systems are usually capable of only gross analysis of substances and are not generally suitable for sensitive analysis. Also, with fluorescent systems, the sample

is illuminated by U.V. radiation to measure the fluoresence and this may cause major problems due to photobleaching.

Broadly stated, according to one aspect of this invention, there is provided a method for the assay, detection, quantification, location or analysis of each of a plurality of substances of interest contained in a sample, which comprises labelling each of said substances with one or more components capable of taking part in a respective distinguishable chemiluminescent reaction. In another aspect, this invention provides a method for the assay, detection, quantification, location or analysis of a sample containing at least two substances of interest which comprises:

(i) treating said sample to form at least first and second complexes, said first complexes being made up of one of said substances, or a respective associated substance, bound or otherwise linked with a first reagent capable of taking part in a first chemiluminescent reaction and said second complexes being made up of another of said substances, or a respective associated substance, bound or otherwise linked with a second reagent capable of taking part in a second chemiluminescent reaction which has emission characteristics

distinguishable from those of said first chemiluminescent reaction;

(ii) subsequently treating said sample containing said first and second complexes to cause said first and second chemiluminescent reactions to occur, and

(iii) observing, sensing, measuring and/or recording the emissions of each of said chemiluminescent reactions.

In yet another aspect, this invention provides a luminescent reagent which comprises a mixture of at least two substances capable of binding or otherwise linking with respective different binding partners, one of said substances being labelled with one or more components capable of taking part in a respective one chemiluminescent reaction and another of said substances being labelled with one or more components capable of taking part in a respective another chemiluminescent reaction of which the emission characteristics are distinguishable from said one chemiluminescent reaction.

We have found that different chemiluminescent labels can be produced which, by appropriate chemical manipulation, possess different characteristics in terms of the speed and wavelength of light emission and that these different labels can be used advantageously in analyte binding systems to permit the substantially simultaneous quantitation of two or more different analytes within

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a single test procedure.

A discussion of non-limiting embodiments of the invention now follows, together with a specific example of an assay procedure, reference being made to the accompanying drawings, in which:-

Figure 1 is a schematic graph showing emission intensity vs. time a typical test procedure according to the invention; and

Figure 2 is a schematic graph showing emission intensity vs. time in an example of procedure according to the invention for the immunochemiluminometric assay for human gouadotrophin and human alpha-fetoprotein.

General Scheme

A and B are the analytes of interest and are each capable of binding more than one antibody molecule so that parallel two-site immunoassays can be set up. A mixture of antibodies capable of binding A and B is coated on to the walls of a test tube. The sample for analysis containing unknown amounts of A and B is added to the tube together with a mixture of soluble complementary antibodies capable of binding to other sites on A and B. The soluble antibodies specific for A and B are labelled with chemiluminescent molecules exhibiting distinguishable characteristics, e.g. fast and slow light emission. Following an appropriate incubation period, two-site immune complexes will be formed on the sides of the tube, the extent of immune complex formation depending on the amount of A and B present. Following removal of unbound substances by spiration of the soluble contents of the tube, the chemiluminescence emission remaining is triggered and then measured in a luminometer. The total number of photons emitted is proportional to the total amount of A and B present.

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However, in this example, the chemiluminescence emission from labelled antibodies specific for A is rapid and complete within one second whereas the emission from labelled antibodies specific for B is much slower and reaches a peak after initiation before decaying over the next nineteen seconds (see Figure 1).
Thus measurement of the photons emitted in two separate time windows of 1 and 19 seconds within an overall measuring time of 20 seconds permits independent quantitation of A and B upon calibration of the system.

<u>Assay Techniques and Analytes</u>

The methods in accordance with the invention may quantify those species of biological interest which are identifiable using single analyte quantitation techniques. The following examples are given with guidance as to the type of binding reaction used. This list is given for example only and does not imply any limitations of the invention:-

1. Immunoassay:
   Drugs, vitamins, steroids, thyroid hormones, peptides, polypeptides, proteins, immunoglobulins, viruses, bacteria, protozoa.
2. Protein binding assays:
   Vitamins, cofactors, enzyme inhibitors.

3. Nucleic acid hybridisation assays:
   Oligonucleotides, polynucleotides, DNA, RNA, oncogenes, microorganisms.
4. Receptor binding assays:
   Progestagen receptors, estrogen receptors, thyrotrophin receptors, thyroid hormone receptors.

Certain pairs of groups of analytes are often measured to get a more complete picture of the biological system or to improve efficiency of testing of the biological system. In such cases the availability of simultaneous, multi-analyte measurement offered by the invention is uniquely advantageous. Examples of such groups of analytes are given below but do not imply limitations of the invention.

1. Hormones:
   Thyroxine/thyrotrophin, lutrophin/follitrophin, adrenocorticotrophin/cortisol.
2. Vitamins and cofactors:
   Vitamin B12/folic acid, 1,25-dihydroxy-cholicalciferol/25-hydroxycholecalciferol.
3. Nucleic acids (from viruses and micro-organisms):
   Neisseria Gonorrhoeae/Chlamydia Trachomatis.
4. Tumour markers:
   Prostate Specific Antigen/Prostatic Acid Phosphatase, alphafetoprotein/carcinoembryonic antigen/chorionic gonadotrophin.

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Labels

The preferred way of associating chemiluminescence activity with the appropriate binding reaction is to chemically or physically couple a component such as a chemiluminescent molecule, capable of taking part in a chemiluminescent reaction,to one of the components of that binding reaction so as to produce a specific labelled reagent. The luminescent reagents according to the present invention will thus include two or more such labelled reagents each carrying a label having different characteristics in terms of kinetic and/or spectroscopic properties. Each of these labelled reagents will have a particular specificity for taking part in a given binding reaction, thus each given binding reaction can be monitored independently even though two or more such reactions are occurring simultaneously. Hence it is possible to quantify, independently and simultaneously, the analytes taking part in these parallel binding reactions.

Different members of a number of classes of chemiluminescent molecules are capable of exhibiting differences in kinetic and/or spectroscopic properties and can hence be used in the invention, including acridinium and related compounds (e.g. phenanthridinium

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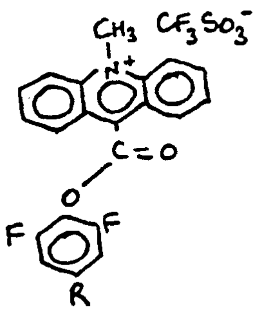
compounds), phthalhydrazides and related compounds (e.g. naphthalhydrazides), oxalate esters and related compounds and also stabilised dioxetanes and dioxetanones. Aryl acridinium esters may be used as labels with appropriate chemical modifications made to produce the desired kinetic and spectroscopic parameters. Some examples of such compounds are given below and do not imply any limitations of the invention.

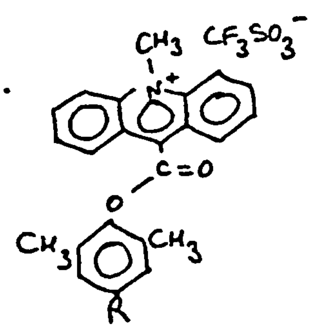
1. Kinetic variation

a.



Duration of light emission under standard conditions = 0.8 seconds

b.



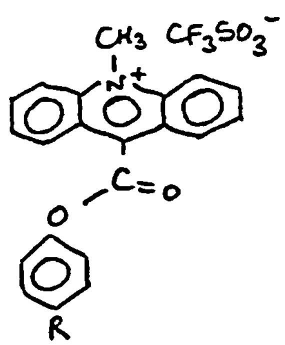
Duration of light emission under standard conditions = 60 seconds

In the acridinium phenyl ester of 1a. the phenyl moiety is substituted with F groups which are electron withdrawing and thus modify the acridinium phenyl ester so that the emission of light occurs over a

relatively short period. In the acridinium phenyl ester of 1b, the phenyl moiety is substituted with CH_3 groups which are electron donating so that the emission of light occurs over a relatively long period. Other electron donating and withdrawing groups may be used.

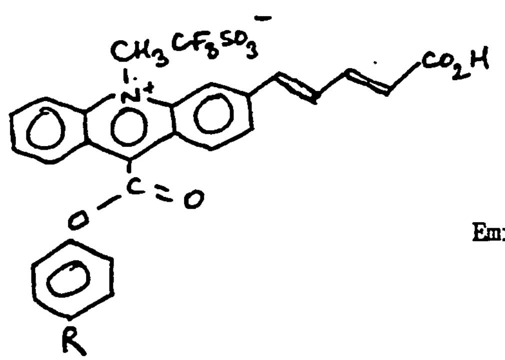
2. Spectroscopic variation

a.



Emission λ max $\sim$ 430 nm

b.



Emission λmax $\sim$ 510 nm

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In 2b, the electronic conjugation of the nucleus has been increased so that the emission radiation is of relatively long wavelength.

In each of the acridinium compounds illustrated above, R is selected to allow covalent coupling to a component of the appropriate binding reaction. Appropriate coupling groups are well described but in this example are selected such that the desired kinetic and/or spectroscopic properties of the molecule are maintained and also that the final labelled reagent is still active in terms of its ability to participate in the binding reaction. Such groups include N-hydroxysuccinimide esters, imidate esters, isothiocyanates and other established active group or groups that can give rise to active groups to facilitate coupling to molecules of biological interest. Preferably these groups are linked to the chemiluminescent moiety by an aliphatic chain of appropriate length.

In a further aspect it is possible to also make use of chemiluminescent reactions which involve energy transfer to a fluorescent acceptor molecule. As an example of this it is possible to label an antibody of one specificity with fluorescein and an antibody of another specificity with rhodamine. These antibodies can be used in simultaneous two-site assays and the end-points determined by introduction of a

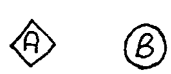
peroxyoxalate chemiluminescence system (hydrogen peroxide/bis-2, 4-dinitrophenyl oxalate). Radiationless energy transfer occurs resulting in the emission of light at two different wavelengths (green-yellow from fluorescein/red from rhodamine), the intensities of the emissions are directly propertional to the amount of the relevant labelled antibody bound in the immunochemical reactions.

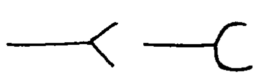
Analyte-binding partners (reagents) and binding reactions

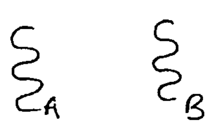
The following schemes represent examples of binding partners or binding reactions that it is possible to use for the determination of concentrations of single analytes which are used currently:

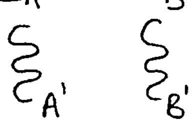
Key:


Solid phase matrix (e.g. coated tube, magnetisable particles)


Different analytes represented by A and B


Antibodies specific for A and B respectively or binding proteins specific for A and B respectively or receptors capable of binding A and B respectively


Oligonucleotide sequences A and B


Complementary oligonucleotide sequences

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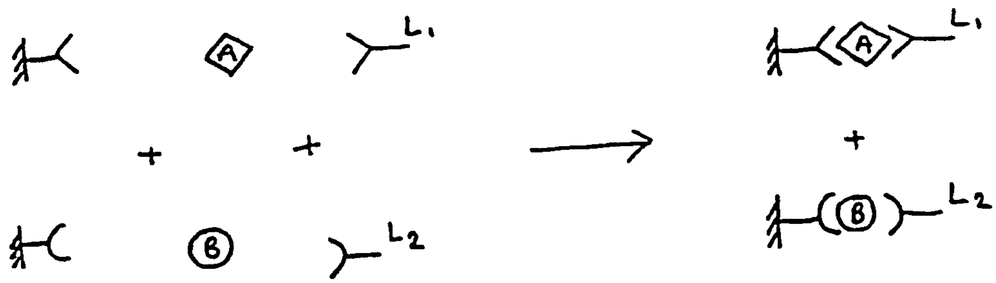
 Binding reagent for double-stranded (recombinant) nucleic acid sequences

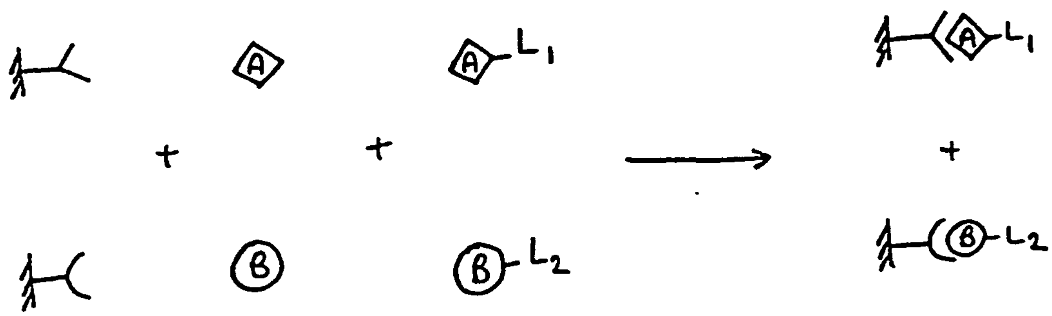
L_1, L_2 Chemiluminescent labels exhibting different reaction kinetics and/or spectroscopic properties respectively.

1a Two-site immunoassays

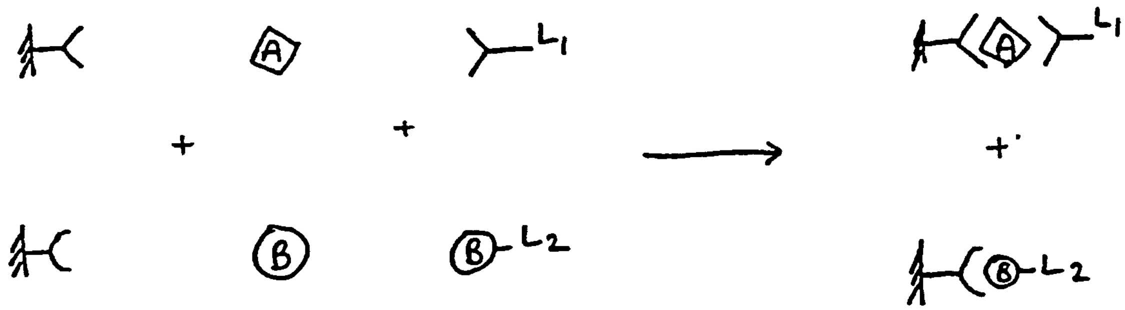


Antibodies of a given specificity for the analyte recognise different parts of the given analyte to permit formation of the two-site immune complex. Excess concentrations of reagent over analyte are used in two-site systems.

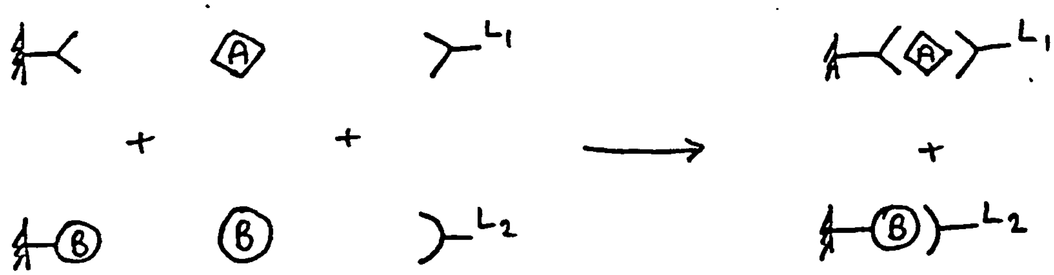
1b Competitive binding immunoassays (labelled antigen)



1c Two-site/competitive binding (labelled antigen) combination.

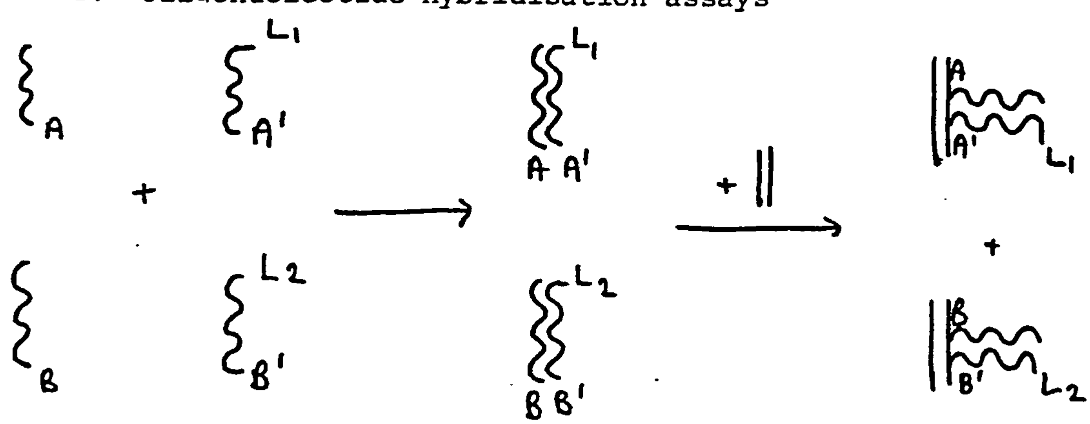


1d Two-site/competitive binding (labelled antibody) combination.



It should be noted that limiting reagent concentrations are used in competitive binding systems.

2. Oligonucleotide hybridisation assays



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The above are all examples of heterogeneous assay systems in which the analyte/binding-partner complex is isolated from uncomplexed material. Additionally it is possible to apply the disclosed systems to homogeneous assays.

Instrumentation

Photon counting equipment may be used for the measurement of light intensity. The sensing equipment should be capable of distinguishing the emissions from the distinguishable chemiluminescent reactions.

1. Kinetic discrimination.

As described in the earlier example, the equipment should be capable of recording measurements of light intensity (preferably as photon counts per unit time) within at least two time frames to permit independent measurements of the intensity arising from slow and fast reactions. In many instances there will be overlap between the two signals which is accounted for by appropriate selection of time frames or by mathematical estimation of the overlap.

2. Spectroscopic discrimination.

Here it is necessary to measure the intensity of two or more wavelengths simultaneously. This can be achieved, eg. by use of the necessary number of photomultiplier tubes each fitted with a bandpass interference filter to permit

measurement of one signal at the exclusion of others. Alternatively a single photomultiplier tube can be used such that the light emitted from the reaction is first passed through a fast scanning spectrometer or filter/chopper system. Synchronisation of the photomultiplier tube output with the scanning or chopping frequency thus permits independent quantitation of the different wavelengths.

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SPECIFIC EXAMPLE

1. Preparation of labelled antibodies

a. 4-(2-carbonylethyl)phenyl-10-methyl acridinium-9-carboxylate fluorosulphonate labelled antibodies to human chorionic gonadotrophin (hCG).

The acridinium label was synthesised as follows: acridinium-9-carboxylic acid (5g) was refluxed with thionyl chloride (15 ml) for 3 hours. The solvent was removed under reduced pressure and the product suspended in anhydrous pyridine (35 ml). Benzyl 4-hydroxyphenylpropanoate (9 nmol) was added and the solution stirred overnight at room temperature. The mixture was subsequently poured into crushed ice/1 M hydrochloric acid (250 ml) and the resulting precipitate filtered, washed with water and dried under reduced pressure. The 4-(2-benzyloxycarbonylethyl)phenyl-9-acridine carboxylate thus obtained was recrystallised from benzene/cyclohexane. 0.46 g of this was dissolved in hydrogen bromide/acetic acid mixture (45/55 w/w, 10 ml) and the solution stirred for 2 h at 50-55°C. The solution was poured into water (100 ml) and the resulting yellow solid, filtered, washed with water and dried under reduced pressure, thence recrystallised from acetonitrile/chloroform to yield 4-(2-carboxyethyl)phenyl-9-acridine carboxylate. N-hydroxysuccinimide (62 mg) was dissolved in dimethylformamide (5 ml) together with 200 mg of the above acridine carboxylate. The mixture was cooled to -20°C and dicyclohexylcarbodiimide (123 mg) added, followed by stirring for 2 h at -20°C, thence overnight at room temperature. One drop of glacial acetic acid was then added and the mixture left for a further 30 min. The

dicyclohexylurea was removed by filtration and the material obtained by evaporation of the liquor was recrystallised from benzene/cyclohexane to yield 4-(2-succinimdyloxycarbonyethyl)phenyl-9-acridine carboxylate. The product (234 mg) was dissolved in anhydrous chloroform (15 ml) and methyl fluorosulphonate (0.5 ml) added. The precipitate which formed after stirring at room temperature for 18 hours was filtered and washed with anhydrous benzene to yield 4-(2-succinimidyloxycarbonylethyl)phenyl-10-methylacridinium-9-carboxylate fluorosulphonate. Mouse monoclonal antibodies (50 µg) raised to human chorionic gonadotrophin were dissolved in sodium phosphate buffer (pH 7.4, 0.1 M, 200 µl) containing 0.15 M sodium chloride. A stock solution was made of the acridinium-succinimidyl ester in acetonitrile (0.5 mg/ml) and 10 µl added to the antibody solution with mixing. After incubation at room temperature for 15 min in the dark, a solution of lysine monohydrochloride (100 µl, 10mg/ml) in the above buffer was added and the mixture left for a further 5 min. The mixture was purified on a column of Pharmacia Sephadex G25-M (30 cm x 0.6 cm) equilibrated and eluted with phosphate buffered saline (pH 6.3, 0.1 M, 0.15 M NaCl) containing 0.1% (w/v) bovine serum albumin and 0,05% (w/v) sodium azide. 0.5 ml fractions were collected and the void volume fractions pooled and stored at 4^{o}C.

b. 4-(2-imidylethyl)-2,6-dimethyl-10-methylacridinium-9-carboxylate dichloride labelled antibodies to human alpha-fetoprotein (AFP).

The acridinium label was synthesised as follows: acridinium-9-carboxylic acid (2.5 g) was refluxed with thionyl chloride (10 ml) for 3 hours. The solvent was removed under reduced pressure and the product suspended in

anhydrous pyridine (25 ml). 2,6-dimethyl-4-hydroxyphenylpropionitrile (1.3 g) was added and the solution stirred overnight at room temperature. The mixture was subsequently poured into crushed ice/1 M hydrochloric acid (250 ml) and the resulting precipitate filtered, washed with water and dried under reduced pressure. The 4-(2-cyanoethyl)-2,6-dimethylphenylacridinium carboxylate thus obtained was dissolved in anhydrous chloroform (15 ml) and methyltrifluormethylsulphonate (0.5 ml) added. The precipitate which formed after stirring overnight at room temperature and addition of diethylether was filtered off and washed with anhydrous benzene to yield 4-(2-cyanoethyl)-2,6-dimethylphenyl-10-methyl-9-acridiniumcarboxylate trifluoromethylsulphonate which was subsequently dissolved (69 mg, 10 ml) in anhydrous methanol. HCl gas was bubbled through the solution under nitrogen, kept at ice temperature for 2 h and left to stand for a further 1 h. The crystals formed were filtered under an atmosphere of dry nitrogen and washed with anhydrous methanol to yield 4-(2-methyloxyimidylethyl)-2,6-dimethylphenyl-10-methylacridinium-9-carboxylate dichloride. Mouse monoclonal antibodies (50 µg) raised to human alpha-fetoprotein were dissolved in sodium borate buffer (pH 9.5, 0.1 M, 200 µl) containing 0.15 M sodium chloride. A stock solution was made of acridinium imidoester in acetonitrile (0.5 mg/ml) and 10 µl added to the antibody solution with mixing. After incubation at room temperature for 30 min in the dark, a solution of lysine monohydrochloride (100 µl, 10mg/ml) in the above buffer was added and the mixture left for a further 15 mins. The mixture was purified as described above.

2. Preparation of solid-phase antibodies

Monoclonal antibodies to human chorionic gonadotrophin and alpha-fetoprotein recognising distinct epitopes to those recognised by the labelled antibodies were coupled to paramagnetic particles using published methods.

3. "Simultaneous" immunochemiluminometric assay for human chorionic gonadotrophin and human alpha-fetoprotein.

Solid-phase antibody suspensions (800 µg/ml) were mixed in equal volumes. Labelled antibody solutions (10 ng/ml anti-hCG, 50 ng/ml anti-AFP) were mixed in equal volumes. Diluent buffer was same as antibody purification buffer (above). Standard mixtures for calibration consisted of solutions containing known concentrations of hCG and AFP in horse serum. 50 µl of patient serum sample were dispensed in duplicate into 12 x 75 mm polystyrene test tube. Standard tubes were set up in duplicate using 50 µl of the appropriate standards. 100 µl of the labelled antibody mixture were added followed by 100 µl of the solid-phase antibody mixture. The tubes were mixed and set aside for 1 h at room temperature. 1ml of wash solution (1.76 g/l sodium dihydrogen orthophosphate, 0.15M sodium chloride, 0.05% (W/v) sodiumazide, 0.5% bovine serum albumin, 1% (v/v) Triton X-100) was added and the tubes placed in a magnetic rack to facilitate sedimentation of the solid phase. The supernatants were decanted to waste and a further 1 ml of wash buffer added followed by mixing of the tube contents. A further sedimentation/decantation step was performed and the tubes placed in a luminometer.

4. Measurement of light intensity

Measurements of light emission were made in a Ciba Corning Magic Lite Analyzer using procedures recommended by the Manufacturer. Manipulation of the software enabled distinct, sequential integration of light intensity with respect to time. Separate integrations were performed in the ranges 0 - 1 and 1 - 2 seconds corresponding to light emission from the hCG and AFP antibodies respectively and hence being proportional to the concentrations of hCG and AFP in the sample. Since some overlap existed due to interference with the hCG signal by the AFP signal, correction was necessary. This was achieved by estimating the AFP contribution to the 0 - 1 second integration using the previously determined relationship of the light intensity at 2 seconds to the integral between 0 and 1 seconds, as can be seen in Figure 2.

[hCG] U/L	Photon Counts	[AFP] kU/L	Photon Counts	Overlap	Corrected hCG Photon Counts
250	1.22×10^6	250	1.14×10^5	3.35×10^4	1.19×10^6
125	8.11×10^5	125	7.4×10^4	2.18×10^4	7.89×10^5
62.5	4.66×10^5	62.5	4.2×10^4	1.24×10^4	4.54×10^5
31.3	2.63×10^5	31.3	2.3×10^4	6.76×10^3	2.56×10^5
15.6	1.33×10^5	15.6	1.3×10^4	3.82×10^3	1.29×10^5
0	3.37×10^3	0	6.6×10^2	1.94×10^2	3.18×10^3

$r = 3.40$

When [hCG] >> [AFP] the residual hCG signal during the second time window made a significant contribution hence perturbing measurement of x. This was not a problem in practice and could be minimised by relative assay optimisation e.g. by manipulation of specific activities of the labelled antibodies.

In situations where accuracy is required even at extreme relative concentrations mutual overlap is accounted for by the use of more complex software capable of iterative or simultaneous equation calculation.

Where overlap is a serious problem alternative labels must be sought that exhibit greater differences in their relative kinetic properties.

CLAIMS

1. A method for the assay, detection, quantification, location or analysis of each of a plurality of substances of interest contained in a sample, which comprises labelling each of said substances with one or more components capable of taking part in a respective distinguishable chemiluminescent reaction.

2. A method for the assay, detection, quantification, location or analysis of a sample containing at least two substances of interest which comprises treating said sample to label one of said at least two substances, or a respective associated substance, with one or more components which can take part in a first chemiluminescent reaction, and to label another of said at least two substances, or a respective associated substance, with one or more components which can take part in a second chemiluminescent reaction, wherein the emission characteristics of said first and second chemiluminescent reactions are distinguishable from each other, subsequently causing said chemiluminescent reactions to occur, and observing, sensing, measuring and/or recording the emissions of each of said chemiluminescent reactions.

3. A method for the assay, detection, quantification, location or analysis of a sample containing at least two substances of interest which comprises:

(i) treating said sample to form at least

first and second complexes, said first complexes being made up of one of said substances, or a respective associated substance, bound or otherwise linked with a first reagent capable of taking part in a first chemiluminescent reaction and said second complexes being made up of another of said substances, or a respective associated substance, bound or otherwise linked with a second reagent capable of taking part in a second chemiluminescent reaction which has emission characteristics distinguishable from those of said first chemiluminescent reaction;

(ii) subsequently treating said sample containing said first and second complexes to cause said first and second chemiluminescent reactions to occur, and

(iii) observing, sensing, measuring and/or recording the emissions of each of said chemiluminescent reactions.

4. A method according to claim 3, wherein said step of treating to form first and second complexes includes reacting said sample with a mixture containing said first and second reagents.

5. A method according to claim 3 or claim 4, wherein said first and second chemiluminescent reactions are caused to occur by effecting substantially simultaneous triggering

of said chemiluminescent reactions.

6. A method according to any preceding claim, wherein the emission characteristics of each of said chemiluminescent reactions are distinguishable in terms of the variation of light emission or radiation intensity with time of the emissions.

7. A method according to claim 6, wherein the intensity of the radiation emitted from said sample is observed, sensed, measured and/or recorded over two different time intervals which may overlap.

8. A method according to any of claims 1 to 5, wherein each of said chemiluminescent reactions emits radiation in a respective different spectral range.

9. A method according to any of claims 1 to 5, which involves at least three chemiluminescent reactions, of which at least two are distinguishable in terms of the spectral emission characteristics and at least two are distinguishable in terms of the variation of light or radiation intensity with time.

10. A method according to claim 8 or 9, wherein the emissions are filtered by respective filtering means responsive to radiation in said different spectral ranges and the filtered intensities of the emissions are observed, sensed, measured and/or recorded.

11. A method according to claim 3 or any claim dependent thereon, wherein at least one of said first and second complexes is made up by a reagent bound to a

substance by one of an immunoassay binding reaction, a protein binding reaction, a nucleic acid hybridisation and a receptor binding reaction.

12. A method according to any preceding claim, wherein each of said chemiluminescent reactions includes a respective component or reagent which is a structural variant of the or each component or reagent associated with the or each other chemiluminescent reactions.

13. A method according to any of the preceding claims, wherein one of the components in at least one of said chemiluminescent reactions is based on acridinium compounds, or structural variants thereof.

14. A method according to claim 13, wherein one of the components in at least one of said chemiluminescent reactions is an acridinium salt alkylated at the ring nitrogen.

15. A method according to claim 14, wherein one of said components in at least one of said chemiluminescent reactions is an acridinium phenyl ester wherein the ester is formed at position 9 of the acridine nucleus.

16. A method according to claim 15, wherein one of said chemiluminescent reactions includes an acridinium phenyl ester wherein the phenyl moiety is substituted with electron withdrawing groups to yield a chemiluminescent reaction in which the emission of light occurs over a relatively short period, and another of said chemiluminescent reactions includes an acridinium

phenyl ester wherein the phenyl moiety is substituted with electron donating groups to yield a chemiluminescent reaction in which the emission of light occurs over a relatively long period.

17. A method according to claim 14, wherein one of said chemiluminescent reactions includes an acridinium salt which, on triggering a said reaction, emits radiation at a relatively short wavelength and wherein another of said chemiluminescent reactions includes an acridinium salt wherein the electronic conjugation of the acridine necleus is increased whereby, on triggering of said another reaction, said acridinium salt emits at a relatively long wavelength.

18. A method according to claim 17, wherein said one chemiluminescent reaction emits radiation of wavelength in the range of from 400 to 500 nm and said other chemiluminescent reaction emits radiation of wavelength in the range of from 500 to 700 nm.

19. A method according to claim 13 or any claim dependent thereon wherein the acridinium compound is modified or further derivatised to permit covalent coupling to a molecule of biological interest.

20. A method according to any of claims 1 to 12, wherein one of the components in at least one of said chemiluminescent reactions is based on one of the following compounds:

(i) phthalhydrazides and related compounds ·

(ii) dioxetanes, dioxetanones and related compounds

(iii) Bis-oxalate esters, related compounds and associated acceptor partners where required.

21. A method for the assay, detection, quantification or location of each of a plurality of different substances of interest contained in a sample, wherein the sample is reacted with a mixture of appropriate binding partners for binding or otherwise linking with respective ones of said different substances, said binding partners each being associated with a respective chemiluminescent labelling system which is distinguishable from the chemiluminescent labelling systems associated with the other binding partners.

22. A luminescent reagent which comprises a mixture of at least two substances capable of binding or otherwise linking with respective different binding partners, one of said substances being labelled with one or more components capable of taking part in a respective one chemiluminescent reaction and another of said substances being labelled with one or more components capable of taking part in a respective another chemiluminescent reaction of which the emission characteristics are distinguishable from said one

chemiluminescent reaction.

23. A luminescent reagent according to claim 22, wherein each of said chemiluminescent reactions are distinguishable from each other in terms of the variation of light intensity with time of the emissions.

24. A luminescent reagent according to claim 22, wherein each of said chemiluminescent reactions emits radiation in a different spectral range.

25. A luminescent reagent according to claim 22 or claim 23, wherein at least one of said substances is capable of binding or otherwise linking in one of an immunoassay binding reaction, a protein binding reaction a nucleic acid hybridisation reaction and a receptor binding reaction.

26. A luminescent reagent according to any one of claims 22 to 25, wherein said labelling components are structural variants of the or each other labelling component.

27. A luminescent reagent according to any of claims 22 to 25, wherein one of the components in at least one of said chemiluminescent reactions is based on acridinium compounds or structural variants thereof.

28. A luminescent reagent according to claim 27, wherein one of the components in at least one of said chemiluminescent reactions is an acridinium salt alkylated at the ring nitrogen.

29. A luminescent reagent according to claim 27, wherein one of said components in at least one of said chemiluminescent reactions is an acridinium phenyl ester wherein the ester is formed at position 9 of the acridine nucleus.

30. A luminescent reagent according to claim 28, wherein one of said chemiluminescent reactions includes an acridinium phenyl ester wherein the phenyl moiety is substituted with electron withdrawing groups to yield a chemiluminescent reaction in which the emission of light occurs over a relatively short period, and another of said chemiluminescent reactions includes an acridinium phenyl ester wherein the phenyl moiety is substituted with electron donating groups to yield a chemiluminescent reaction in which the emission of light occurs over a relatively long period.

31. A luminescent reagent according to claim 27, wherein one of said chemiluminescent reactions includes an acridinium salt which, on triggering of said reaction, emits radiation at a relatively short wavelength and wherein another of said chemiluminescent reactions includes an acridinium salt wherein the electronic conjugation of the acridine nucleus is increased whereby, on triggering of said another reaction, said acridinium salt emits radiation at a relatively long wavelength.

32. A luminescent reagent according to claim 31,

wherein said one chemiluminescent reaction emits radiation of wavelength in the range of from 400 nm to 500 nm and said other chemiluminescent reaction emits radiation in the range of from 500 nm to 700 nm.

33. A luminescent reagent according to claim 27, or any claim dependent thereon, wherein the acridinium compound is modified or further derivatised to permit covalent coupling to a molecule of biological interest.

34. A luminescent reagent according to any one of claims 22 to 25, wherein at least one of said chemiluminescent reactions is based on one of the following compounds:

(i) phthyhaldydrazides and related compounds

(ii) dioxetanes, dioxetanones and related compounds

(iii) Bis-oxalate esters, related compounds and associated acceptor partners where required.

35. A method or reagent for the assay, detection, quantification, location or analysis, of substances of biological interest substantially as hereinbefore described.

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